second nucleic acid, wherein said second **[DNA]** nucleic acid specifically integrates at the site of FRT recombination **[an FRT within the genome of said mammalian cell]** and combines with said first **[DNA]** nucleic acid, wherein the combination of said first nucleic acid and said second **[DNAs,]** nucleic acid prevents expression of the first gene of interest.

REMARKS

The novel site-specific recombination system of the present invention provides an artisan with the ability to target the integration of transfected DNA to a **specific** chromosomal site in a mammalian host cell at **frequencies exceeding** those of both random and other site-specific integration systems. Additionally, this novel recombination system allows for **immediate confirmation** and analysis of the recombination event. Applicants' recombination system is distinctive in its **precision** and **predictability**, providing methods which enable an artisan to **routinely create or disrupt** a **functional** translational reading frame at an intended site of integration.

By the present communication, claims 25, 26, 28, 42-44 and 48 have been amended to define Applicants' invention with greater particularity. In addition, the specification has been amended to indicate the continuing status of the present application and also to correct an obvious typographical error. No new matter is introduced by the amendments. Support for the amendments is found throughout the specification of the application as originally filed.

Accordingly, claims 25, 26, 28, 42-46 and 48 are currently under examination.

I. REJECTIONS UNDER 35 U.S.C. § 112, FIRST PARAGRAPH

Claims 25, 26, 28, 42-46 and 48 stand rejected and the specification is objected to under 35 U.S.C. § 112, first paragraph, as the specification allegedly fails to provide a reasonable written description, enablement and best mode for practicing the claimed invention. This rejection is respectfully traversed.

Applicants respectfully disagree with the Examiner's assertion that the specification does not disclose how the initial FRT site is precisely inserted (Paper No. 24, page 2, lines 17-18). The procedure for transfecting eukaryotic DNA into eukaryotic cells is standard practice, well known to those of ordinary skill in the art. It is also known in the art that the site of integration of DNA into a cell's chromosome depends upon the particular specie of eukaryotic cell. For example, when transfecting yeast DNA into yeast cells, it is known that integration most often occurs at a chromosomal site that is homologous to the DNA segment being introduced, i.e., homologous recombination. In contrast, integration of foreign DNA in mammalian cells often results in recombination/integration at nonspecific chromosomal locations, i.e., non-homologous recombination. Therefore, integration of the **initial FRT** site is not targeted. Applicants' invention requires only that the initial FRT site integrate within a chromosome. Once the initial FRT site is chromosomally integrated, this chromosomally integrated FRT, then, serves as the **target site** for subsequent

integration of specific nucleic acid sequences or genes of interest. The precise site of integration can be readily determined using standard detection assays, i.e., Southern blot analysis, histochemical screening, and the like.

In all instances, Applicants provide substantial enablement for the practice of the present invention. For example, Applicants teach the introduction of an initial FRT site into a cell by way of a first transfection step. See Example I, at page 17 through page 22, line 4. Applicants further teach that the initial FRT, once chromosomally integrated, serves as a target site for the precise integration of **subsequent** nucleic acids having at least one FRT site.

In addition, in another embodiment, Applicants teach linearization of the pFRTßGAL reporter plasmid construct with restriction enzyme XbaI. An XbaI insert containing a neomycin cassette flanked on each of the 5' and 3' termini with half FRT sites is ligated to the linearized plasmid. The plasmid half FRT sites combine with the insert half FRT sites and form two complete but, **modified** FRT sites on the 5' and 3' termini of the neomycin cassette. These modified FRT sites serve as substrates for FLP recombinase enzyme. The insertion of the neomycin insert, moreover, **disrupts** the translational reading frame of ß-galactosidase, preventing expression of the ß-galactosidase gene product. This is reporter plasmid, pNEOßGAL.

The reporter plasmid is linearized and transfected into a cell. Once inside the cell, the reporter plasmid randomly integrates into a chromosome of the cell. If pFRTßGAL is chromosomally integrated, the cell will constitutively express

ß-galactosidase and carry one chromosomally integrated FRT site which serves as a substrate for FLP recombinase and as a target for the precise integration of subsequent nucleic acids. Precisely targeted integration of a nucleic acid at this integrated FRT site results in the **loss** of ß-galactosidase activity.

If pNEOßGAL is chromosomally integrated, the cell will have G418 resistance and carry two chromosomally integrated FRT target sites. A vector expression construct encoding FLP recombinase, (e.g. pOG44) can be transfected into this cell. Unlike the reporter plasmid, the expression vector does not integrate into a chromosome of the cell, it is only expressed transiently and will not be passed on through mitosis. Once inside the cell, the expression vector expresses FLP recombinase enzyme/protein. The FLP recombinase enzyme seeks its substrate, i.e., the two modified restored FRT sites on the pNEOßGAL plasmid chromosomally integrated in the mammalian cell. When the FLP recombinase comes in contact with the integrated FRT sites, it catalyzes the modified sites to recombine. The recombination event excises the neomycin cassette and restores the original minimal FRT site. Recombination results in the restoration of the translational reading frame of ß-galactosidase, allowing for expression of ß-galactosidase. The resulting cell line, designated CVNEOßGAL/E, carries a **single** integrated FRT target site. Precisely targeted integration of a nucleic acid at this integrated FRT site results in the loss of ß-galactosidase activity.

In view of the above remarks, reconsideration and withdrawal of this rejection and objection, therefore, is respectfully requested.

Claims 25, 26, 28, 42-46 and 48 stand rejected under 35 U.S.C. § 112, first paragraph, as the disclosure allegedly fails to enable precise targeting of the second DNA. This rejection is respectfully traversed.

Clearly, Applicants have detailed the methods of the invention in the specification such that a person of ordinary skill in the art could reproduce and practice Applicants' invention. Therefore, Applicants respectfully submit that the claims under examination are, indeed, enabled in the specification such as to allow one of ordinary skill in the art to prepare the claimed subject matter without undue experimentation, employing standard techniques. Therefore, it is respectfully submitted that Applicants' specification unquestionably sets forth the best mode of practicing the invention contemplated by Applicants at the time of filing.

Applicants' invention provides for modifications, such as targeting nucleic acids to a specific site (an integrated FRT), wherein integration of a nucleic acid will produce a functional gene, produce a functional hybrid gene or prevent expression of a functional gene. The present invention can be practiced using standard techniques well known to skilled artisans.

Thus, in an embodiment of the present invention, Applicants provide cell lines, CVNEOßGAL/E, which carry a single

chromosomally integrated plasmid pNEOßGAL which consists of two chromosomally integrated FRT target sites flanking a neomycin resistance cassette. ß-galactosidase expression is blocked by the presence of the neomycin cassette. Transient expression of FLP recombinase is shown to catalyze recombination between the integrated FRTs, excision of the neomycin cassette and result in the creation of a single chromosomal copy of pFRTßGAL. The resulting cell line constitutively expresses ß-galactosidase and carries a single integrated FRT site which serves as a substrate for FLP recombinase, and also as a precise target site for integration of specific nucleic acid sequences or gene(s) of interest. A specific nucleic acid sequence or gene(s) of interest can be cotransfected into this cell line along with an expression construct encoding FLP recombinase. FLP recombinase targets the integrated FRT site and induces recombination of the integrated FRT site with the FRT site(s) flanking the nucleic acid sequence or gene(s) of interest. Integration of such nucleic acid sequence or gene(s) disrupts the translational reading frame of ß-galactosidase and prevents expression thereof. The precision of targeted integration can be readily determined using a simple assay which detects ß-galactosidase activity.

In another embodiment, pNEOßGAL, the reporter plasmid described above, is used to construct a targeting vector, (e.g. pOG45) which has the neomycin cassette and 3' FRT site from the reporter plasmid. The targeting vector is cotransfected along with the expression vector into the CVNEOßGAL/E cell line, which carries a single chromosomally integrated copy of pFRTßGAL. Once inside the cell, the expression vector expresses FLP recombinase enzyme. The FLP recombinase enzyme seeks its substrates, i.e., the chromosomal and extrachromosomal FRTs. The extrachromosomal

FRT of the targeting vector recombines with the chromosomal FRT stably integrated in the CVNEOßGAL/E cell line. The FLP-induced recombination enables the targeting vector (carrying a gene of interest) to integrate at a predetermined site, i.e., the FRT site, forming two complete, but modified FRT sites. The newly integrated gene of interest (in this example, neomycin) confers neomycin resistance upon the cell and also disrupts the translational reading frame of the ß-galactosidase coding sequence and prevents expression thereof. Thus, two readily identifiable events confirm precise targeted integration as presently claimed.

In view of the above remarks, reconsideration and withdrawal of the rejections and objection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

II. REJECTIONS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 25, 26, 28, 42-46 and 48 stand rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse this rejection.

It is respectfully submitted that the Examiner's concerns have been rendered moot by the amendments to claims 25, 26, 28, 42-46 and 48 submitted herewith. Specifically, in claim 25, the "first/initial" FRT site (chromosomally integrated) is distinguished from the "second" nucleic acid containing at least one FRT. Amended claim 25 requires integration of the initial FRT, **before** subsequent nucleic acids containing at least one FRT

site can be targeted thereto. Claims 25 and 26, as amended, clarify the role of the FLP recombinase as the catalyst of recombination between respective FRT sites, i.e., chromosomally integrated FRTs and extrachromosomal FRTs. Applicants' specification identifies the FRT site as a "substrate for the [FLP] recombinase." (See, page 16, lines 11-13, and page 17, lines 5-7).

Further, in response to the Examiner's query regarding "the existence of naturally occurring FRT sites", the specification identifies FLP recombinase and its respective substrate, FRT, as derived from a yeast strain, in particular *Saccharomyces cerevisiae* (page 11, lines 1-34). This enzyme-substrate combination is not endogenous to, or naturally occurring in, mammalian cell lines. Accordingly, the presence of a "naturally occurring FRT site" in a mammalian cell is improbable.

The Examiner's concerns regarding the recitation of "within a functional portion" have been rendered moot by the amendment of claim 43 provided herewith. Thus, the functional portion is further defined as being within the protein coding sequence of a gene of interest. (See, page 17, lines 28-32, and page 18, lines 17-20 of Applicants' specification.)

In view of the above amendments and remarks, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested.

III. REJECTIONS UNDER 35 U.S.C. §§ 102(b) or 103

Claims 25 and 28 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by, or in the alternative, under 35 U.S.C. § 103 as allegedly being obvious over Golic et al., *Cell* 59:499-509 (1989). These rejections are respectfully traversed.

Applicants' invention, as defined by claims 25 and 28, requires integration of a nucleic acid into the chromosome of a **mammalian** cell at a specific target site, i.e., FRT, that is stably integrated in said chromosome. In contrast, Golic et al., discuss recombination between FRTs in **insect** cells, Drosophila melanogaster. Anticipation requires the presence, in the cited reference, of **all** elements of an invention as set forth in the claims. The Golic et al. reference, therefore, does not anticipate the present invention as defined by claims 25 and 28. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 102(b) is respectfully requested.

Applicants respectfully disagree with the Examiner's assertion that

> it would have been obvious from the disclosure . . . to expect the process to function in other organisms which are higher eukaryotes (page 499, left column) where mammalian cells (page 499, right column) are known higher eukaryotic cells.

(Paper No. 24, page 4, lines 15-18.) The Examiner points to three independent statements in Golic et al. that allegedly support the aforementioned assertion. However, a reference which allegedly renders an invention unpatentable must be taken in its **entirety**, including those portions which argue **against**

obviousness. Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc., 230 USPQ 416, 420 (Fed. Cir. 1986). It is impermissible within the framework of § 103 to pick and choose from a reference only so much of it as will support a conclusion of obviousness to the exclusion of other parts necessary to a full appreciation of what the reference fairly suggests to one skilled in the art. Id. at 419.

It is respectfully submitted that the statements cited by the Examiner as allegedly rendering the present invention obvious, are unrelated, have been taken out of context, and do not teach or suggest Applicants' invention. For example, the Examiner cites the Introduction section, on page 499, left column, wherein Golic et al. compare the predictability and ease of recombination in yeast cells, i.e., homologous recombination, with recombination in **higher eukaryotes**, which is "**rare** and **difficult** to control." Thus, it is apparent that Golic et al. teach **away from** homologous recombination in eukaryotic systems higher than yeast.

Another citation specifically noted by the Examiner, also in the Introduction section, on page 499, right column, refers to a reason for choosing *Drosophila* as the host system. Specifically, Golic et al. acknowledge another known recombination system (e.g. *Cre lox*), that has previously been shown to effect recombination between *lox* recombination target sites integrated into the chromosomes of **yeast** (*S. cerevisiae*); and also to effect recombination between *lox* recombination target sites on **extrachromosomal plasmids** in **mammalian** cells (citing Sauer). Herein, Golic et al. note that although other systems effect recombination between chromosomally integrated targets in

yeast cells, the recombination effected in mammalian cells is **not** between chromosomally integrated targets. The last sentence of the Discussion section, on page 507, right column, concludes that although the FLP system has only been tested in *Drosophila* (i.e., a single species of **insect**), "we **expect** that it will work in **other organisms** as well". Golic et al. do not, however, specifically identify what is intended by reference to "other organisms". It is submitted that the teachings of Golic et al. suggest that "other organisms" comprise organisms **less complex** than mammalian host systems. Thus, the combination of the specific citations selected from Golic et al. by the Examiner, when taken in context, teach away from utilizing mammalian cells for FLP recombination. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103 is respectfully requested.

IV. REJECTIONS UNDER 35 U.S.C. § 103

Claims 25, 26, 28, 42-46 and 48 stand rejected under 35 U.S.C. § 103, as allegedly being unpatentable over Sauer ('317) taken with Golic et al. This rejection is respectfully traversed.

Applicants' invention, as defined by all of the claims, distinguishes over the art by providing methods for integrating and recombining DNA at a precise target, a specific, predetermined site, FRT, stably integrated in the genome of a mammalian host cell. Applicants' conditional recombination system utilizes a site-specific recombinase, FLP, from *Saccharomyces cerevisiae* and its recombination substrate/targets, FRTs, for manipulation of chromosomally integrated nucleic acid

sequences in mammalian cells. Sauer ('317) does not teach manipulation of chromosomally integrated nucleic acid sequences in mammalian cells. In contrast, the recombination system taught by Sauer utilizes a protein from bacteriophage P1, *Cre*, and its recombination target, *lox*, to effectuate recombination and integration of DNA into the chromosomes of the **non-mammalian yeast** species *S. cerevisiae* and into target sites on **extrachromosomal plasmids** in **mammalian** cells. Applicants' recombination system efficiently catalyzes recombination of FRT sites and precisely targets the **chromosomal** integration of nucleic acids or gene(s) of interest into a mammalian cell. In contrast, Sauer's recombination system effects recombination between target sites on **extrachromosomal plasmids** in mammalian cells. Sauer only teaches chromosomal integration and recombination in **non-mammalian** yeast cells. Sauer ('317) does not, either alone, or in combination with Golic et al., teach or suggest Applicants' invention.

Applicants have discussed the Golic et al. reference above. The Golic et al. discussion presented above applies equally with respect to the current rejection. Golic et al. do not teach or suggest a method for **precisely** targeting a specific FRT site integrated in the genome of a mammalian cell. Golic et al. do not teach or suggest means to rearrange and recombine specific FRT target sequences, wherein the recombined targeted sequences allow a nucleic acid or gene of interest to integrate at that chromosomal site and express a functional gene product. Golic et al., either alone, or in combination with Sauer, neither teaches nor suggests the present invention.

The rationale set forth by Golic et al. for using *Drosophila* as the host cell for the FLP recombination system teaches away from using a mammalian host system. Clearly, the justification of Golic et al. for using *Drosophila* as the host for the FLP recombination system would not motivate a skilled artisan to modify Sauer ('317), as required for the practice of the present invention. For example, (on page 499, last paragraph, left column continuing onto right column) Golic et al. teach away from using mammalian host cells and toward using *Drosophila* host cells. Specifically, Golic et al. teach that there is precedent for a yeast nuclear protein with sequence-specific binding properties working in *Drosophila* (citing Fischer et al., "GAL4 Activates Transcription in *Drosophila*", *Nature* 332:853-856 (1988)). In addition, Golic et al. refer to other recombination systems and respective host cells, specifically the *Cre lox* system as utilized in yeast and mammalian host cells (citing Sauer), and point out **shortcomings** associated therewith, i.e., only extrachromosomal recombination in mammalian cells and the tendency for unwanted recombination of target *lox* sites. In view of the state of the art, i.e., since the yeast protein, GAL4, has evolved to function in a higher eukaryotic cell (than yeast), i.e., *Drosophila*, Golic et al. propose that it is reasonable to assume that another yeast protein, FLP, would also function in a higher eukaryotic cell (than yeast), i.e., *Drosophila*. However, Golic et al. neither teach nor suggest that the yeast protein, FLP, functions in a higher eukaryotic cell (than *Drosophila*), i.e., mammalian cells. Indeed, the complexity of the mammalian genome renders the *Drosophila* genome elementary in comparison. It was clearly unexpected, at the time of the present invention, that the yeast protein, FLP, could efficiently

catalyze recombination between FRT target sites chromosomally integrated into the genome of a mammalian cell.

Claims 25, 26 and 28 stand rejected under 35 U.S.C. § 103, as allegedly being unpatentable over Sauer taken with Golic et al., and further in view of Palmiter et al. This rejection is respectfully traversed.

Further reliance on Palmiter et al. is unable to cure the deficiencies of the primary references. Indeed, a discussion of the Palmiter et al. secondary reference is not necessary in view of the fact that the combination of the Sauer and Golic et al. primary references neither teach nor suggest the present invention. As discussed above, the combination of the primary references teaches away from efficient chromosomal integration and recombination of yeast target sites, FRTs, in mammalian host cells.

The Palmiter reference does not cure the inadequacies of the primary references. The combination of the primary references suggests that a protein from an organism of lower evolutionary origin **cannot** recombine chromosomally integrated targets in highly evolved organisms having substantially more complex and developed genomes, i.e., mammalian host cells. The *Cre lox* recombination system effected recombination on extrachromosomal plasmids in mammalian cells, **not** on chromosomally integrated targets. When the primary references suggest that certain proteins are ineffective or inefficient in mammalian cells, the teachings of Palmiter et al., i.e., introduction of **functional** genes into mammalian germlines, are irrelevant. Accordingly, the combination of Sauer taken with

Golic et al., and further in view of Palmiter et al. does not teach or suggest the present invention.

In view of the above remarks, reconsideration and withdrawal of the rejection of the claims under 35 U.S.C. § 103, is respectfully requested.

V. SUMMARY

In view of the above amendments and remarks, reconsideration and favorable action on all pending claims is respectfully requested. If any questions or issues remain, the Examiner is invited to contact the undersigned at the telephone number set forth below so that a prompt disposition of this application can be achieved.

Respectfully submitted,

5/4/94
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